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INSTRUMENTAL AND SENSORY ANALYSIS OF THE ACTION OF CATHEPTIC ENZYMES ON FLAKED AND FORMED BEEF

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Abstract

Texture profile analysis, Instron punch and die testing, laser diffraction measurements and scanning electron microscopy (SEM) were used to evaluate the effects of catheptic enzymes on flaked and formed beef. Although salt (NaCl) and sodium tripolyphosphate (TPP) have often been used to improve the textural quality of flaked and formed beef, the catheptic enzymes used in this study were shown to be as effective as NaCl/TPP, and in many cases more effective, in improving the textural characteristics of this product. Instron punch and die and scanning electron photomicrographic analysis showed that the enzyme and NaCl/TPP samples were quite similar; however, laser diffraction measurements of sarcomere lengths were significantly longer for the enzyme treated samples. Also, various textural parameters including amount of connective tissue were significantly improved by catheptic enzyme treatment.

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Introduction

The purpose of comminution (i.e. flaking and fabricating) has been to upgrade less acceptable cuts of meat (Seideman, 1982) by mechanically breaking down connective tissue, which is a major contributor to toughness, and thus improve textural and other sensory characteristics (Anon., 1973). Flaking, as in the Comitrol process (Urschel Laboratories, Inc., Valparaiso, IN 46383) is accomplished by revolving meat inside a circular cutting head and forcing the meat through columns of vertical knives. Next, the flaked meat is mixed (sometimes under vacuum) with binding adjuncts (usually a combination of sodium triphosphate [TPP] and salt [NaCl]) and then, after tempering and pressing, which re-forms the flakes into a solid log of meat of suitable cross-section area, the meat is sliced to an appropriate thickness to produce a steak-like product (Fenters and Ziemba, 1971).

Although the meat is more tender after flaking, the incorporation of different cuts of meat, each with varying amounts of connective tissue, into the same product, may cause differences in textural uniformity.

Catheptic enzymes, which are lysosomal proteases, have been shown to degrade myofibrillar Z-bands and sarcolemma at post-mortem pH (Eino and Stanley, 1973a,b; Robbins and Cohen, 1976; Robbins et al., 1979) as well as collagen (Etherington, 1974; Barrett, 1972). Not only do these enzymes play a significant role in aging-related tenderization, but they have also been shown to increase the textural uniformity of pre-cooked, freeze-dried meat (Cohen et al., 1979).

To date, the effects of catheptic enzymes on flaked and formed processed meat have not been determined; therefore, in this paper we present data on the action of these enzymes, compared with NaCl and TPP, on the texture of flaked and formed meat.

Materials and Methods

Preparation of Spleen Extract Containing Catheptic Enzymes

As described by Robbins and Cohen (1976), fresh bovine spleen obtained from a local slaughterhouse was cut into small pieces, added to 3X

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its volume of acetone and stirred for one hour at 5°C. Upon decantation, the residue was air-dried to remove the acetone. Next, the residue powder was suspended in 4X its volume of water, stirred for one hour, and then filtered through cheese cloth. The residue was then discarded and the filtrate adjusted to pH 3.2 with 2N HCl. Following centrifugation of the filtrate at 30,000 x g for 15 minutes, the pH of the supernatant was adjusted to 7.0 with 2N NaOH and the solution centrifuged again at 30,000 x g for 15 minutes. After the supernatant was dialyzed against distilled water to eliminate the chloride ion, it was freeze-dried. Prior to use as a freeze-dried powder, the specific catheptic enzyme activity was assayed by the methods of Keilova and Tomasek (1976) for cathepsin D and Barrett (1972) for cathepsin B. For cathepsin D, one unit of enzyme activity is equivalent to an increase of 1.00 absorbance unit at 280 nm in 1 min. using acid denatured hemoglobin as the substrate. For cathepsin B, one unit of enzyme activity is equivalent to 1.00  $\mu$ mole of benzoylarginine-p-nitroanilide (BAPA) hydrolyzed in 1 minute.

#### Flaked and Formed Beefsteak Production

Boneless USDA Utility square cut beef chucks obtained from a local slaughterhouse were held overnight in a tempering box at between -2°C and 0°C. The next morning the meat was flake-cut with an Urschel Comitrol Model 3600 using a 2-J030750-D cutting head (about 2 cm openings) and then divided into three 4.5 kg portions. The flake cut meat was randomly assigned to one of three treatments: (1) no additives (control); (2) 0.5% salt (NaCl) and 0.25% sodium tripolyphosphate -  $\text{Na}_5\text{P}_3\text{O}_{10}$  (TPP); (3) a freeze-dried spleen extract containing catheptic enzymes at a level of approximately 8 gm of the dry powder per 4.5 kg (10 lbs.) of meat.

All the samples from each treatment group were mixed under vacuum in a Keebler ribbon-type mixer for 15 minutes and then stuffed into polyethylene tubing. Next, the meat logs were frozen to approximately -18°C and then tempered to between -2°C and 0°C. Then the meat logs were pressed at 500 psi in Die #452 (approximately 10.8 cm square with rounded corners) into a ribeye shape using a Bettcher Press Model #70. The formed logs were sliced with a Bettcher Cleaver Model #39 to a thickness of approximately 12.7 mm (0.5 in.) and weight of 170 gms (6 oz.), separately wrapped with freezer wrapping paper (this packaging was employed since storage stability was not a factor in this study), frozen to -18°C and stored until used.

#### Instrumental Texture Analysis

Samples were cooked from the frozen state on a preheated Farberware Grill Model 455N for approximately 12 minutes per side, to an internal temperature of 68°C. A 13 cm long copper-constantan thermocouple probe was inserted into the partially cooked steak after it was turned the second time. The end of the probe was placed as close to the geometric center as possible. Because the thermal gradients perpendicular to the steak surface were very large, the location of the thermocouple was critical. It was necessary, however, to do some probing with the thermocouple to assure that all parts of the steak had reached

desired temperature. After cooking, all samples were allowed to reach room temperature (22°C) before instrumental analysis was carried out. Measurements were made using the Instron Universal Testing Machine Model TTDM with a punch and die test cell (Segars et al., 1975). The deformation of the meat produced a force-deformation curve from which the shear-stress ( $\gamma$ ) parameter was derived. Shear stress ( $\gamma$ ) =  $F_t/\pi dT_0$  where  $F_t$  = maximum shear force,  $d$  = punch diameter and  $T_0$  = sample thickness. Since the shear force is tangential to the cylindrical wall of the punch and is directed along its axis, the area over which the force is distributed =  $\pi dT_0$ . Shear stress was calculated in Newtons/cm<sup>2</sup> and statistical significance at the  $p < 0.05$  level was determined using the matched t-test.

#### Texture Panel Analysis

Samples were cooked on a Farberware Grill Model 455N to an internal temperature of 68°C, turning once. After cooking, samples were halved and placed on heated ceramic dishes for evaluation by the panel. Five trained texture profile panelists evaluated each of the three test samples at each of eight test sessions. Occasional absences of panelists resulted in a total of 36 evaluations for each sample.

Samples were evaluated for seven textural attributes. Each attribute was rated at a specific time during the mastication process. These times and the definitions of each attribute were as follows:

1. During partial compression with molar teeth: (a) Springiness - extent to which the sample returns to its original shape and thickness after slight compression with the molar teeth.
2. During first bite with molar teeth: (b) Hardness - perceived force required to compress the sample between the molar teeth; (c) Cohesiveness - degree to which the sample holds together upon biting.
3. During mastication: (d) Chewiness - force required to reduce the sample to a consistency ready for swallowing when chewed at a constant rate; (e) Moisture/oil content - amount of water and/or oil perceived in the sample during mastication; (f) Cohesiveness of the mass - degree to which the sample holds together as a single mass during mastication; (g) Amount of connective tissue (gristle) in the sample.

Samples were presented in random order within each session and panelists were instructed to rate all three samples on one attribute before proceeding to another. Each attribute was rated on a seven-point category scale of intensity, where 1 = slight and 7 = extreme.

The statistical difference in means of the enzyme vs. control and enzyme vs. NaCl/TPP sample for each of the seven attributes was tested by a matched t-test with a two-tailed probability. Criterion for significance was set at the 0.05 probability level. In addition, analysis of variance with linear contrasts was performed using ANOVA and Newman-Keuls tests.

#### Sample Preparation for Scanning Electron Microscopy

Pieces of meat approximately 1 cm<sup>3</sup> were cut

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from each sample and immersed in 2.5% glutaraldehyde in phosphate buffer pH 5.8 overnight at 4°C. The next morning they were rinsed twice for two hours each in buffer, postfixed for one hour in 1% OsO<sub>4</sub>, and they dehydrated in a graded ethyl alcohol series (30%, 50%, 70%, 90%, 95%, 100%) for 30 minutes each. From absolute ethyl alcohol, the samples were critical-point dried with CO<sub>2</sub> using a Balzers CPD 010 critical-point drier. They were then mounted on aluminum stubs using double-sided tape, sputter coated with AuPd in a Commonwealth Scientific mini-coater and, finally, examined in a Coates and Welter 100-2 Scanning Electron Microscope at between 15 and 20 kV.

### Sarcomere Length Measurements

Muscle fiber bundles were teased from those samples which were being prepared for scanning electron microscopy and immersed in a few drops of the fixative used above on a depression slide. Ten individual fibers were dissected from the bundles and mounted in a drop of fixative which had been pipetted onto a clean glass slide. A coverslip was placed over the drop and excess fluid blotted from around the edges.

The slide was then mounted onto a holding device on an optical bench so that when a fiber lay in the path of a laser beam from a Spectra Physics Laser (HeNe - 632.8 nm) diffraction bands were produced on a screen which was placed exactly 100 mm from the sample. The formula used to convert the distance between the zeroth and first order diffraction bands to sarcomere length is as follows:

$$d = \frac{(632.8 \times 10^{-3} \text{ } \mu\text{m}) (D)}{S}$$

where the sarcomere length (d) equals the wavelength of laser light expressed in micrometers times the distance (D) from the muscle fiber to the screen on which the diffraction bands are formed, divided by the distance (S) in millimeters between the zeroth and first order diffraction bands. The matched t-test was used to calculate the significance at the  $p < 0.05$  level.

## Results

### Instrumental Texture Analysis

In Table 1 the shear stress values for the control, NaCl/TPP and enzyme treated samples are compared. The shear stress value for the control samples was significantly higher than for the NaCl/TPP and enzyme treated samples; however, there was no significant difference between the NaCl/TPP and enzyme samples. Both the enzyme and NaCl/TPP samples had lower standard deviation values than the control which indicates perhaps more uniformity.

### Texture Panel Analysis

In Table 2, sensory texture profile data for enzyme-treated flaked and formed beef is compared with control samples and samples prepared with NaCl/TPP. The enzyme treated samples were significantly less chewy, had less cohesiveness of the mass and had less connective tissue than the controls. The enzyme samples were also significantly less springy, less chewy, had lower moisture/oil content, less cohesiveness of the mass and had significantly less connective tissue than the NaCl/TPP samples.

Table 1. Comparative Mean (N=36) Shear Stress Values<sup>1</sup> of Cooked Control, Salt (NaCl)/Sodium Tripolyphosphate (TPP) and Catheptic Enzyme Treated Flaked and Formed Meat.

Samples	Shear Stress (Newtons/cm <sup>2</sup> )	±	Standard Deviation
Control	14.627	±	3.364
NaCl/TPP	11.210	±	2.076
Catheptic Enzyme	10.699	±	1.799

<sup>1</sup>There were significant differences ( $p < 0.05$ ) between the NaCl/TPP vs. Control and Cathepsin vs. Control, but none between the NaCl vs. Cathepsin.

Table 2. Sensory Texture Profile Analysis<sup>1</sup> of Cooked Control, Catheptic Enzyme and Salt (NaCl)/Sodium Tripolyphosphate Treated Flaked and Formed Beef.

	Control	Enzyme	NaCl/TPP	Results of ANOVA F-value, dF, probability
Springiness	2.53 <sup>b</sup>	2.72 <sup>b</sup>	3.86 <sup>a</sup>	F=14.53, dF=70, $p < 0.05$
Hardness	3.88	3.86	3.64	F=0.47, dF=70, $p < 0.05$
Cohesiveness	3.47	3.31	2.83	F=2.54, dF=70, $p < 0.05$
Chewiness	4.70 <sup>a</sup>	4.14 <sup>b</sup>	4.86 <sup>a</sup>	F=6.32, dF=70, $p < 0.05$
Moisture/Oil Content	3.94 <sup>b</sup>	3.86 <sup>b</sup>	4.75 <sup>a</sup>	F=11.84, dF=70, $p < 0.05$
Cohesiveness of the Mass	3.61 <sup>a</sup>	3.06 <sup>b</sup>	3.72 <sup>a</sup>	F=3.95, dF=70, $p < 0.05$
Amount of Connective Tissue	4.44 <sup>a</sup>	3.28 <sup>b</sup>	4.33 <sup>a</sup>	F=14.22, dF=70, $p < 0.05$

<sup>1</sup>Differences in means (N=36) were evaluated by Neuman-Kuels contrast tests. Mean values in the same row with different superscripts are significantly different at the 0.05 probability level.

### Sarcomere Length Measurements

The laser diffraction-sarcomere length measurements are given in Table 3. When statistical comparisons were made between the control vs. enzyme and NaCl/TPP vs. enzyme samples, it was found that treatment with the enzyme produced significant sarcomere lengthening. The NaCl/TPP samples had significantly longer sarcomere lengths than the control; however, they were, in turn, shorter than the enzyme treated samples.

Table 3. Comparative Mean (N=36) Laser Diffraction-Sarcomere Length Values<sup>1</sup> of Cooked Control, Salt (NaCl)/Sodium Tripolyphosphate (TPP) and Catheptic Enzyme Treated Flaked and Formed Beef.

Samples	Sarcomere Length (μm)	±	Standard Deviation
Control	1.42	±	0.13
NaCl/TPP	1.54	±	0.12
Catheptic Enzyme	1.69	±	0.08

<sup>1</sup>The differences between all of the samples were significant at the  $p < 0.05$  level.

### Scanning Electron Microscopy

In the uncooked control (Fig. 1) one can see small globular structures (arrow) covering the meat surface. Also present are a few larger structures (G) which might be caused by the coalescence of smaller ones. When the control was cooked, many more larger globular structures appeared on the surface (Fig. 2).

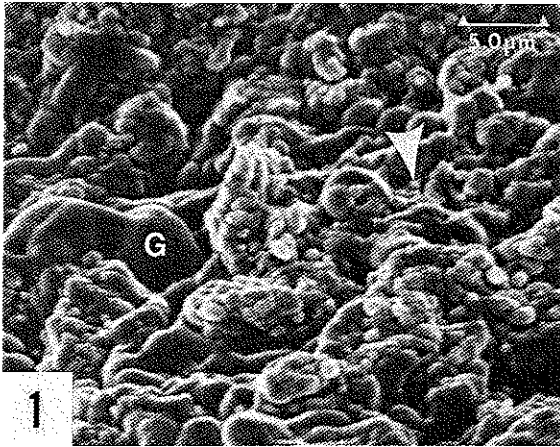


Fig. 1. Uncooked Control. Globular particles (arrow) are found over the surface of muscle fibers. The large globular structure (G) was probably produced by the fusion of many smaller ones during mixing.

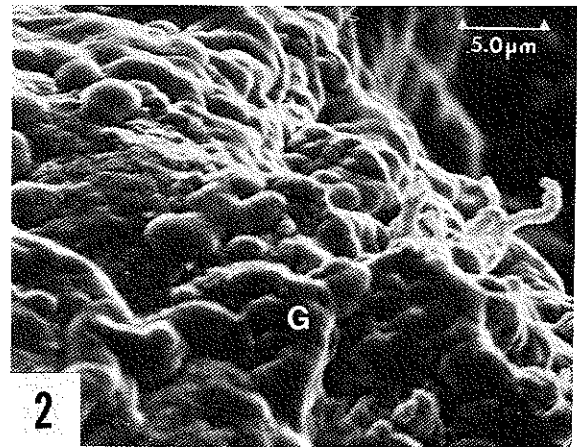


Fig. 2. Cooked Control. Fusion of the globules (G) is probably caused by coagulation when the meat is heated.

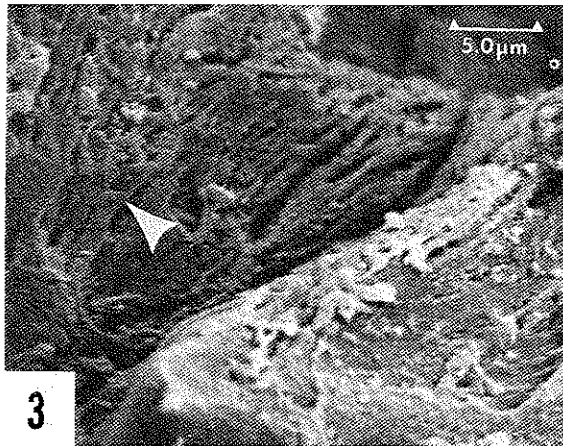


Fig. 3. Uncooked Samples Treated With NaCl and TPP. Some degradation has occurred exposing the underlying myofibrils (arrow).

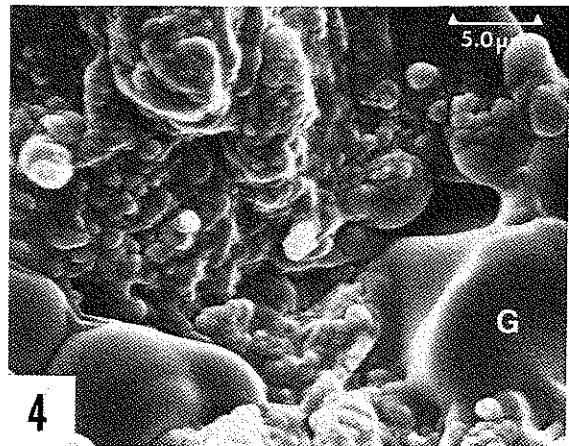


Fig. 4. Cooked Samples Treated With NaCl and TPP. Heat-induced coagulation produces very large globules (G).

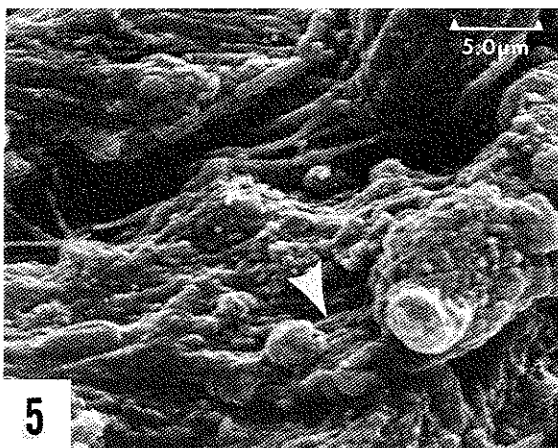


Fig. 5. Uncooked Sample Treated With Catheptic Enzyme. Degradative action on the sarcolemma has exposed the myofibrils (arrow).

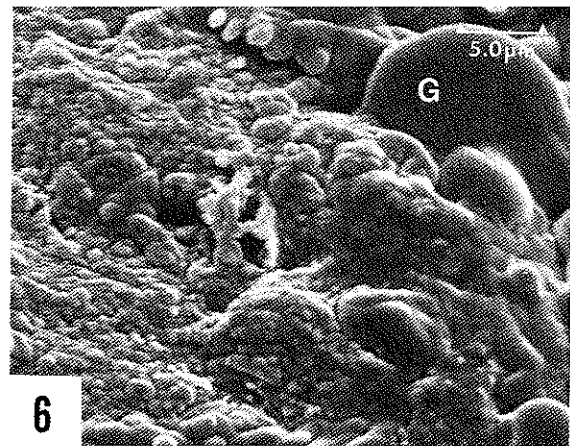


Fig. 6. Cooked Sample Treated With Catheptic Enzyme. Cooking causes the fusion of coagulated globules (G).

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Typical myofibrillar bundles (arrow) are evident in the uncooked NaCl/TPP sample (Fig. 3); however, when the NaCl/TPP samples were cooked, very large globular structures (G) as well as smaller ones appear on the surface (Fig. 4).

The uncooked enzyme-treated sample (Fig. 5) appears somewhat similar to Fig. 3, and, upon cooking, the very large globular structures (G), which are quite similar to those in Fig. 4, appear in Fig. 6.

### Discussion

Although there were significant sarcomere length differences between the NaCl/TPP and enzyme-treated samples, the results of the shear measurements showed no such differences. In work on the effect of catheptic enzyme on stored freeze-dried beef, Cohen et al. (1979) found that not only did those samples treated with cathepsins have longer sarcomere lengths than the controls, but that there was an inverse correlation between shear stress and sarcomere length values, i.e. the higher the sarcomere length, the lower the shear stress. It appears from the data presented in Tables 1 and 3 that a similar relationship exists for both the NaCl/TPP and enzyme-treated flaked and formed meat.

As seen in the micrographs, the differences in the surface appearance of the uncooked control samples, as compared to the uncooked NaCl/TPP or enzyme samples suggests that treatment with NaCl/TPP or catheptic enzymes (West et al., 1974) results in degradation of the sarcolemma, and leads to increased exudation of myofibrillar and sarcoplasmic proteins. Furthermore, the exudated substances coalesce upon heating.

Booren et al. (1981) found that mixing causes the release of some sarcoplasmic proteins with some coagulation prior to heating. Since NaCl/TPP (Theno et al., 1978) and cathepsins make the membranes more permeable, substantially more heat coagulation of the proteins occur when these samples are heated (Jones et al., 1977; Bard, 1965). Schaller and Powrie (1972) suggested that the granular material seen on the surface of bovine muscle fibers which had been heated was coagulated sarcoplasmic protein. Also, in a study of the ultrastructure of heated bovine muscle, Cheng and Parrish (1976) stated that the globular structures found on the surface of heated muscle could be either disintegrated collagen fibers or coagulated sarcoplasmic proteins. This could explain the very large globular structures seen in the micrographs.

Mandigo (1974) found that when NaCl alone is added to restructured meat, the flavor and texture were significantly improved, and when NaCl was combined with TPP, Huffman (1979) found a reduction in cooking loss. Panel evaluation data presented by Schwartz and Mandigo (1976) indicated that addition of NaCl and TPP improved most sensory characteristics.

When our texture panel evaluated the textural parameters of flaked and formed meat treated with NaCl/TPP or catheptic enzymes, they found the springiness of the enzyme samples to be significantly less than that of the NaCl/TPP samples. Since springiness is an undesirable attribute of

these steaks, the reduction in springiness effected by the enzyme is a beneficial result of this treatment.

Neither the hardness nor cohesiveness values were significantly different between the control, enzyme and NaCl/TPP samples. On the other hand, chewiness values were significantly different, with the enzyme-treated samples requiring less total force to chew when compared with the NaCl/TPP group or with the controls.

Both the moisture/oil content and cohesiveness of the mass were significantly higher in the NaCl/TPP samples. NaCl has been shown to increase the juiciness of restructured meat (Mandigo, 1974) and NaCl, together with polyphosphate, have increased the binding of beef rolls (Pepper and Schmidt, 1975).

According to Siegel and Schmidt (1979), a combination of NaCl and phosphate increased the ability of myosin to bind by solubilizing the sarcoplasmic proteins. They concluded that NaCl acts to solubilize myosin whereas phosphate causes the dissociation of actomyosin. Ford et al. (1978) showed that "crude" myosin, either alone or in combination with sarcoplasmic protein has the potential for binding pieces of meat without the addition of NaCl.

Finally, the connective tissue, which is a major contributor to meat toughness (Anon., 1973), was perceived to be significantly less in the enzyme-treated samples compared with the controls and the NaCl/TPP samples. Therefore, the use of catheptic enzymes, which degrade connective tissue as well as myofibrillar proteins, perhaps combined with polyphosphate, which is synergistic with cathepsin D (Watabe et al., 1979), might improve textural characteristics sufficiently to permit the use of less costly cuts of meat at significant monetary savings.

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#### Discussion With Reviewers

E. A. Davis: Why did the authors use a matched t-test in analyzing their data? An analysis of variance (ANOVA) with a least significant difference test applied, where appropriate, would have been more accurate and powerful. Was there a statistical reason for not doing this?

R. W. Mandigo: Would not data have been better analyzed and would not greater confidence in the analytical work resulted if the data were analyzed by linear contrasts, not by paired t-tests?

Authors: The use of a matched t-test is appropriate since the obtained alpha level for the tests was 0.02. With only 3 groups of samples and 3 apriori comparisons, the actual overall error rate for any of the sensory variables has a Bonferroni upper bound of slightly over 0.05. We deemed this acceptable in our calculations. Because of the reviewers' questions, we recalculated some of our data (Table 2) using repeated means ANOVA and Newman-Keuls contrast tests and found that the conclusions regarding significant differences did not change from our matched t-tests.

S. C. Seideman: You claimed in your paper that the enzyme-treated steaks had less organoleptically detectable connective tissue. Do you believe the cathepsins actually break down connective tissue?

Authors: Based on Etherington (1974) who discussed the degradative action of catheptic enzymes on connective tissue and Robbins and Cohen (1976) who show the degradation of connective tissue surrounding myofibrillar bundles, we do believe that at least one of the cathepsins breaks down connective tissue.

R. W. Mandigo: Please interpret the likelihood that the small and large globules in the electron micrographs with respect to whether they are fat or protein?

H. D. Geissinger: How do the authors know that the structure marked with an arrow in Fig. 1 (uncooked control sample) is a small globule of sarcoplasmic protein? Could the structure also be part of endo- or perimysial connective tissue, or even a preparation artifact?

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E. Varriano-Marston: Interpretation of micrographs was highly speculative. The authors would have been better off doing cryosections and light microscopy to see what was going on. As an example, I do not see how the authors can identify the sarcoplasmic proteins in Fig. 1. How do they know these globular structures are not other proteins or fat?

Authors: Based on the work of Schaller and Powrie (1972), Cheng and Parrish (1976), Booren et al. (1981) and others, we concluded that the majority of the globules were of sarcoplasmic origin, that the globules in the uncooked samples were formed as a result of mixing and that the globules in the cooked samples were formed by heat-induced coagulation. Since the proportion of cathepsin B and D in the freeze-dried powder was higher than the other cathepsins present, there was a greater likelihood that the globules were composed of sarcoplasmic (or other connective tissue) or myofibrillar proteins, since cathepsin B and D are specific for these components. Since each of the other cathepsins in the powder has a specific activity against various other structures or components within and surrounding the muscle fiber, it is possible that some of the globules might be composed of some other material. However, we think it is unlikely that the globules are composed of fat or that they are preparation artifacts.

S. C. Seideman: If restructured steaks were made from larger chunks instead of flaked beef, would you expect the cathepsins to overly tenderize the chunk by breaking down myofibrillar proteins?

Authors: Our experience has shown that catheptic enzymes act selectively without causing the typical mushy texture which papain causes when it is allowed to remain on meat's surface for an extended time. It would probably take longer to tenderize larger chunks than flaked beef.

S. C. Seideman: Since most enzymes including cathepsins are temperature and pH dependent, would you expect different results with different mixing temperatures and pH's and what would you expect the results to be?

Authors: At 70°C cathepsin D is completely inactivated within 2 minutes and at 50°C it is 50% inactivated within 40 minutes; however, the threat of bacterial contamination would prohibit mixing at a temperature much higher than 4°C unless precautions were taken. Since the pH of aging meat is approximately 5.3, the use of a pH much lower or higher than that would probably yield an unsatisfactory product.

S. C. Seideman: Except for the 15 minute mixing period, the restructured steaks were always frozen. Do you think the 15 minute mix is long enough or can cathepsins also work during the cooking procedure?

Authors: Perhaps a longer mixing time would stimulate the release of more catheptic enzymes, but time saving is essential to any food preparation process. As mentioned in an answer above, complete inactivation of cathepsin D occurs at 70°C within 2 minutes and 50% inactivation at 50°C, so it is probable that the enzyme activity continues while meat is being cooked, until 70°C is reached.

R. W. Mandigo: Are catheptic enzymes approved for use by either USDA or FDA for use with flaked and formed meat?

Authors: We have not applied for approval from either of these agencies to use catheptic enzymes with flaked and formed meat. Since these enzymes are naturally occurring lysosomal enzymes found within most tissues in the human body, including the liver, spleen and certain white blood cells, I do not expect any problem. After all, various plant proteases including papain, which is a major component of a well known commercial meat tenderizer has FDA and USDA approval.

R. W. Mandigo: What is the chemical composition with respect to moisture, fat and protein content of each of the treatments, and, if they are different, what was the impact of those differences on the interpretations presented?

Authors: The percent H<sub>2</sub>O, fat, protein and ash of the samples were as follows: control, 56.24% H<sub>2</sub>O, 25.98% fat, 16.72% protein and 0.84% ash; cathepsin, 57.44% H<sub>2</sub>O, 24.33% fat, 17.18% protein and 0.85% ash; NaCl/TPP, 57.62% H<sub>2</sub>O, 23.36% fat, 17.12% protein and 1.45% ash. Except for the higher ash content of the NaCl/TPP samples and the slightly lower fat values of the cathepsin and NaCl samples, all the other values appear to be similar and thus had no impact on our conclusions.

Editor: From where can one obtain Huffman, D. L. (1979) and Mandigo, R. W. (1974) references?

Authors: These proceedings are available from National Live Stock and Meat Board, Chicago, IL 60611.